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epithelium

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THE IRON CONTENT OF MAMMARY EPITHELIUM

by

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INTRODUCTION

The popular concept of the etiology of spontaneous adenocarcinoma in the mammary glands of mice, outlined by Bittner (1942), embraces three known factors:

1. The genetic constitution of the individual
2. The mammary tumor agent
3. The hormonal stimulation

It is probable that undiscovered influences play an important role in tumor production for the present concept does not adequately explain all the phenomena.

Following the discovery of Mendel's paper on heredity, at the turn of the century, effort was made to ascertain the genetic influence if any on mammary tumor production in mice. It was noted by Gaylord (1906) and Bashford (1909) that certain colonies of mice had an endemic tumor incidence. Bashford concluded there was no indication that heredity played a part in tumor production; Gaylord hypothesized that mammary tumors were the result of bacterial spread in dirty cages. Leo Loeb (1907) disproved Gaylord's hypothesis and concluded that mammary tumor incidence was affected by heredity. Tyzzer (1909), although his conclusions was not statistically proven, was under the impression that "There is a greater liability to the development of tumors in the off-spring of parents with tumors than

in the off-spring of animals which are throughout life free from tumors." Murray (1911) showed that 18-20% of his animals from cancerous ancestry developed tumors compared to 8.6% for animals from non-tumorous parents.

Little (1916) realized the necessity of developing isogenic strains of mice. The individual variations noted in an experiment would then be the result of environmental factors controlled by the experimenter. Little (1934, 1941 a and b) demonstrated a relationship between mammary tumor development and a known inherited single factor coat color trait, lethal yellow. This is the best absolute proof of the genetic influence on mammary adenocarcinoma in mice. Since that date Bittner (1947) and many others have published work attempting to demonstrate an inherited susceptibility in mice to tumors of the mammary glands.

Lathrop and Loeb (1918) discovered in several crosses between animals with tumors and those without, that the incidence of tumors in the hybrid appeared to follow that of the mother. It was not until the establishment of inbred strains of mice that this finding was interpreted as evidence of a new extra-chromosomal factor. The Staff of the Jackson Memorial Laboratory (1933) repeated the work of Lathrop and Loeb using isogenic strains of mice and proved the pre-

dominance of a material influence. Korteweg (1934 and 1936) confirmed these findings and postutated three possibilities to explain the transmission of extra-chromosomal influence from mother to off-spring:

1. Via the cytoplasm of the ovum.
2. Via the placenta during intra uterine life.
3. Via the mother's milk.

Bittner (1936) foster nursed high tumor strain young on low tumor strain mothers and vice versa and showed that a powerful extrachromosomal factor was present in high tumor strain milk because in each case the tumor incidence of the young approached that of the foster mother. This milk factor (the extrachromosomal agent) has features which suggest the action of a virus or related agent. Andrewes (1939) has produced evidence to show that the latency of the tumor process could be accounted for by the action of a latent virus. Kahler and Bryan (1943) have shown, by ultracentrifugation of tumor extracts, the presence of two entities, (molecular weights of 3 and 5 million), both of which are Bials positive (proof of pentose) and both of which on ultra-violet spectrographic studies show a high percentage of nucleic acid. Cell free filtrates of tumors containing these entities cause the production of mammary adenocarcinoma. These factors strongly suggest a virus like body.

The obvious dependence of the mammary gland upon ovarian secretions, among others, has long been recognized. Lathrop and Loeb (1913) noted a greater mammary cancer incidence in breeding as compared with virgin mice and almost none in the males. These facts were later confirmed by Bittner (1935) using homozygous mouse strains. Cori (1926 and 1927) and Murray (1927) reported that ovariectomy at sixteen days of age radically reduced mammary tumor incidence in mice. Lacassagne (1932 and 1936) and Gardner (1935) have produced mammary adenocarcinoma in male mice following injections with oestrogens. The males, under this artificial stimulation, had a tumor incidence comparable to that of the breeding females of the same strain, thus linking the estrogenic with the virus and genetic factors.

The mode of action of estrogen in tumor processes is unknown. It may act as a synergist or catalyst directly with the milk agent or it may cause the development of the mammary gland in the proper kind and degree necessary for the other carcinogenic factors to operate. That these three factors; genetic, hormonal and viral individually are incapable of tumor production in the mouse is recognized and it is only through the combined action of all three that neoplasm

results. Twombly (1940), Bittner (1942), Shimkin and Andervont (1941).

Shimkin et al (1941) and Huseby and Bittner (1946) are of the opinion that the milk factor does not alter the architecture of the mammary gland structure of mice. Van Gulik and Korteweg (1940) reported that the presence of the milk influence caused a greater degree of duct arborization. Gardner (1939) did not mention this difference but showed that localized adenomatous mammary nodules were in general characteristic of high tumor strains. Shimkin et al (1941) has shown that adenomatous nodules plus a lack of uniformity in mammary gland structure were seen in mice receiving the milk factor and were not seen in off-spring of reciprocal crosses when the mother was of low tumor stock.

It becomes apparent that there is no general agreement on the action of the milk and genetic factors on the mammary gland because subjective methods have been used to make quantitative estimations of gland development and architecture. It was felt that an accurate quantitative method of measuring mammary epithelial development would be a valuable aid in the investigations of the effect of the known carcinogenic factors on the mammary glands. A survey of the literature has shown that the only methods used for measur-

ing alveolar mammary development have been histological and these are only roughly quantitative and permit no accurate comparison. The monumental and time consuming works of Cole (1938), Fekete (1938) and Gardner and Hill (1936) depend entirely on histological methods and although their work is painstaking in its detail the subjective factor associated with their techniques allows little basis for correlation with other work.

Because of the great lack of quantitative data on the stages of mouse mammary gland development it was decided to attempt to correlate histological findings of the mammary gland to some substance which could be expressed in a quantitative way. The substance chosen was a peculiar intra epithelial deposit of granular iron. Gibson (1930) in a study of the development of the mammary gland in the mouse incidentally mentioned the occurance of an intra epithelial pigment.

Shultz (1933) reported finding stainable intra epithelial granular iron in the mammary glands of male and female rats and female mice. These iron inclusions in the mouse and rat appeared at the time of sexual maturity and remained throughout the animals life. A curious disappearance of the iron granules occured late in pregnancy but they were restored im-

mediately upon removal of the young or during the later stages of lactation. Shultz believed that this iron was not related to the milk function of the gland. The appearance at puberty, the disappearance during pregnancy and recurrance during lactation suggest the possibility that the mammary gland of the mouse has a secondary sexual activity akin to that of the apocrine sweat gland epithelium in the human. The mouse has no apocrine glands corresponding to human axillary glands and Shultz considered the possibility that the iron in the mammary gland then functioned in the production of sexually attractive odoriferous substances. Bonser (1936) refers to the presence of iron containing granules lining the acini in the mammary glands of mice that had been treated for a number of weeks with estrone. The reference was incidental to a study of the effect of estrone on the mammary glands of mice.

Rawlinson (1949), Rawlinson and Hankinson (1948), investigated the occurence of iron containing granules in the normal mammary epithelium surrounding spontaneous adenocarcinomas in mice. These inclusions were found only in cells which had not become neoplastic.

Rawlinson (1950) used the Gomori technique in whole mounts to show that the stainable epithelial iron inclusions found in the mouse mammary gland were

stored principally in the end bulbs and alveoli. Moreover the amount of stainable iron present was correlated histologically to the degree of alveolar development. It was considered that if a chemical method of iron analysis could measure this stainable intra epithelial iron which had already been correlated to gland structure, then the result would be a quantitative method of measuring gland development. Because cancer research has great need of quantitative data especially as regards the pre-tumor stages an attempt was made to develop a method of measuring the stainable intra epithelial mammary iron of mice and to use this as an indication of gland development. Such a technique could be of value in assessing the effect of the three known main factors in the development of mouse mammary cancer viz: genetic, viral and hormonal.

A related problem which was also studied was the relationship of the human to the mouse mammary gland in the light of the phenomenon of iron retention. Human skin has iron containing glands called Apocrine sweat glands. Homma (1926), Richter (1933). There are also areas in the human female mammary gland which appear to have a similar structure to these apocrine sweat glands and like them to contain iron. Pathologists are divided in two general groups in their

opinions as to the origin of this pale mammary epithelium. One school believes these pale areas found in the human mammary gland represent actual or altered sweat glands while the other considers they are derived from ordinary mammary tissue. (Reviews by: Lee, Pack and Scharnagel 1933, Higginson and McDonald 1949, Lancet Editorial 1949 and Lendrum 1945). Any further evidence of iron containing epithelial cells in the human mammary gland would affect the question. Bunting (1948) found lipoid and iron patterns typical of apocrine sweat glands in the pale epithelium of the human female breast and concluded that these areas represented apocrine sweat glands. This opinion is not shared by Speert (1942)or Geschickter (1943) who approached the problem from a different standpoint. Consequently a study was made of the occurrence of iron in the mammary glands of human males with the object of throwing light on this question.

METHODS AND MATERIALS

a. Mouse Mammary Tissue

The animals consisted of groups of male, virgin and female mice that had been used as breeders but that had been isolated from the male for at least four

weeks and had not been pregnant or suckled during that period. Two strains of highly inbred animals were used: 1. C_3H and 2. C_{57} Black.

The C_3H strain was originated in 1920 by Strong (1935) by mating a male from Little's strain dba to a female obtained from Bagg's colony of albinos and the wild type black agouti segrants were selected. Bittner obtained the line from Strong in 1931. C_3H breeder females have a cancer incidence of 95%, virgin females 63%. Huseby and Bittner (1946).

Strain C_{57} Black was originated in 1921 by Little by mating a heterogenous male and female obtained from Lathrop. The black off-spring were selected as C_{57} Black and inbreeding has been carried on for fifty generations. C_{57} Black is a low mammary tumor strain, less than 1% of breeding stock producing spontaneous mammary adenocarcinoma. Heston (1945). Dr. John Bittner kindly furnished the C_3H and C_{57} stock used to found this colony.

Until December 1949 the diet of all animals used in this laboratory consisted of Victor Fox Chow but because the animals were not breeding the diet was then changed to Ralston Purina fox checkers. Pregnant animals were removed from the breeding pens and were isolated from the males until their litters were dead or weaned. (30 days of age). Careful records were maintained of

the animals age, breeding history, and the age of the appearance of tumors.

Because the amounts of iron were small it was found necessary to use acid washed (iron free) glass probes and splinters for actual gland and lymph node dissections, to prevent iron contamination. Chemically pure reagents were used in all cases, the nitric acid and ammonium hydroxide were redistilled to lower their iron content. All glassware used in the determinations was washed with a commercial detergent, rinsed in tap water, allowed to soak for an hour in concentrated C.P. hydrochloric acid, rinsed three times in tap water and three times in redistilled iron free water.

The animals were weighed, killed by decapitation and then skinned using a ventral or belly incision. The skin with the adherent mammary glands was pinned out on a cork board. The lymph nodes (a large one centrally placed and often a smaller one at the dorsal extremity) associated with each of the inguinal mammary glands were shelled out and discarded. The paired inguinal glands were chosen for chemical analysis because they may be dissected from the hide as units and because they contain a negligible amount of muscle tissue. The two inguinal glands were then placed together in weighing bottles and their combined wet weights recorded.

The hide with the remaining mammary glands was fixed in 10% neutral buffered formalin (Lillie 1948) for histological study using whole gland mounts (Rawlinson 1950) stained by the Gomori (1936) technique. The rationale was as follows: After fixing in 10% neutral buffered formalin for twenty-four hours, the axillary glands were scraped from the hide using an iron free porcelain spatula. The pieces of tissue were then soaked in redistilled water for at least one hour to remove the fixative and then were placed in a freshly made mixture containing equal parts 10% potassium ferrocyanide and 20% hydrochloric acid for a period of from twenty to thirty minutes. Several washings in redistilled water followed by 70% ethyl alcohol eliminated the staining mixture. Immersion for two hours or more in each of 95% ethyl alcohol, absolute ethyl alcohol, oil of origanum and xylol caused dehydration and clearing of the tissue. The sheets of muscle tissue, associated with the axillary glands, were dissected free from the gland by means of fine probes used under a dissecting microscope. The glands were then flattened by compression between two pieces of glass and then mounted in piccolyte. Microscopic assessment was then made of the stained intra epithelial iron.

The paired inguinal glands whose wet weights had been recorded were wet ashed according to the method of Jackson (1938) and the iron content of the resultant fluid was estimated colorimetrically using αα dipyridyl (Hill, 1931) following the technique of Kitzes et al (1944). The method was comprised of the following operations:

1. DISSOLUTION OF THE TISSUE: The glands were placed in a 30 ml. pyrex microkjeldahl flask with one ml. concentrated sulphuric acid and three ml. nitric acid and heated over a micro burner until the gland tissue was dissolved. Care at this time must be observed to prevent "frothing over" of the solution.

2. 3. INCINERATION AND DILUTION: One ml. 70-72% perchloric acid was added, boiling was continued and if charring occurred nitric acid was added drop by drop until the solution cleared. The yellow color of the solution at this time is due to the formation of nitrosyl sulphuric acid (Sandell) which will interfere with the later iron estimation. Heating was always continued until a water clear solution was obtained (all nitrosyl sulphuric acid was driven off) which was then cooled and diluted with eight to ten ml. redistilled water.

4. NEUTRALIZATION: One drop of indicator (0.2% P-nitrophenol in water) was added followed by concen-

trated ammonium hydroxide until the solution just became yellow (pH 7).

5. BUFFERING AND REDUCING: Two ml. acetic acid-acetate buffer (pH 4.5) were added followed by a strong reducing agent (2 drops Eastman's commercial grade thioglycolic acid).

6. COLOR DEVELOPMENT: One ml. αα dipyridyl (0.2% in 5% acetic acid solution) was added and the resulting red colored solution transferred to a volumetric flask and made up to 25 ml. with redistilled water. The solution was allowed to stand at least 1/2 hour which ensured complete color development. The final pH was 4.3.

7. READING THE LIGHT TRANSMITTANCE: The solution was transferred to a 25 ml. Coleman cuvette and the light transmittance read in a Coleman Junior spectrophotometer at a wave-length of 515 mμ*. A blank solution which contained all the materials and which had been processed simultaneously was also read to determine the amount of reagent iron that had been added during the experiment.

*A spectral transmittance curve was plotted (see graph 1) using a solution of 1.5γ of iron per ml. of solution in excess αα dipyridyl. 515 mμ was found to be the wave length at which ferrous dipyridyl has minimum light transmittance and therefore greatest sensitivity.

8. RECORDING AND RESULTS: The light transmittance expressed as a percentage was recorded and the concentration of iron expressed in gamma per ml. of solution was read from the standard graph*

b. Human Mammary Tissue

The human tissue used in this experiment was post mortem material procurred from 21 male subjects of various ages (Table 10). In each case an incision was made to the depth of the fascia covering the pectoralis major so that the block of tissue contained an elliptical area including where possible the nipple, areola and about 1/4 to 3/4 of an inch of peri-areolar skin. The tissue was fixed in 10% neutral buffered formalin dehydrated, imbedded in celloidin, and thick serial sections, usually 100 μ but some were 200, 300 or 500 μ, were cut. Alternate sections were stained for iron using the Gomori mixture and cleared without counterstain. The others were stained with alum hematoxylin to show the tissue morphology. The sections were mounted serially in piccolyte and examined.

*This graph had been made by plotting on semi logarithmic paper the percentages of light transmittance against their known concentrations of iron in ferrous dipyridyl. Ferrous dipyridyl follows the Beere-Lambert law so this graph is a straight line. (see graph 2)

RESULTS

1. Histological:

The glands from the axillary region which were stained for iron in whole mounts were graded on the basis of their stainable intra epithelial iron content as +, ++, or +++ according as the iron appeared to be scant moderate or great in amount. A total of 61 males, made up of two strains C_3H and C_{57} Black, had only the most rudimentary duct system and no stainable intra epithelial iron was ever demonstrated in them or their end bulbs but the lymph nodes associated with the inguinal mammary glands always gave a positive iron reaction.

Table 1 summarizes the results obtained from virgin and resting breeder C_3H and C_{57} Black female mice, a total of 90 animals. Thirteen C_{57} Black virgins showed no blue granular intra epithelial mammary iron, merely a dark green coloration of the ducts which was believed due to minute concentrations of soluble iron too small in amount to be precipitated in the granular form. The histological method of measuring iron in the mammary glands of the mouse is relatively crude but the results of the above study indicate definitely that for either strain the mammary glands of breeder females contain more stainable iron than those of the virgins.

2. Chemical

The results of the chemical iron assay for male mice of the two strains are shown in Table 2. The small standard deviations and standard errors of the mean indicate that the total iron in the male fat pad for any strain used is a relatively constant amount. Statistical analysis shows that there is a high degree of probability that a true difference exists between the means of the two groups because the P value is less than 1%. Further analysis demonstrates a coefficient of correlation between mammary pad weight and total mammary pad iron of:

0.72 (P less than 1%) for C_3H males

0.41 (P less than 2%) for C_{57} males

These highly significant coefficients indicate that for either strain the total iron varies as the weight of the mammary pad of the male mouse. The formulas for regression curves were calculated and the graphs plotted. Graph 3. Since the male gland has been shown histologically to contain no stainable intra epithelial iron then the regression curve will show at a glance the average amount of iron likely to be present in the blood, enzymes and tissue in a mammary fat pad of given size in the particular strain concerned.

The female mouse mammary gland presents a peculiar

anatomical picture for its epithelium is contained within an extensive fat matrix. The weight of the female mammary epithelium when compared to that of the total mammary pad is very small and for practical purposes the weight of the gland itself may be considered negligible because the fat and water of the pad comprise about 95% of its total weight. Shaw (1950). The total iron in the female is a combination of stainable iron, found only in the alveolar cells of the gland epithelium, plus the diffuse non staining protein bound iron (hemoglobin, enzymes and fixed tissue iron) associated with the fat matrix. This latter fraction, for the purpose of measuring stainable intra epithelial iron, must be excluded. The fat matrix of the pad in the female is structurally the same as that in the male. The total iron of the male pad is a measure of the protein bound iron associated with a given weight of mammary pad in any strain. It will thus be a fair measure of the blood and enzyme iron in the matrix of the female gland pad of corresponding weight and strain. Because the male regression curve shows at a glance the average iron content for any given weight of mouse mammary fat, the weight of the female gland need only be located on the appropriate line of the graph and the average amount of protein bound iron may be read.

If this is subtracted from the figure for the total mammary pad iron, the result will be the amount of stainable intra epithelial iron contained in the mammary gland of the female.

Table 3 indicates the histological and chemical results obtained from eighteen C_3H virgin females, from 25-50 weeks of age, arranged in descending order of intra epithelial iron as determined on the above basis. Table 4 shows similarly twenty-eight breeder C_3H mice arranged in identical manner. Comparison of the results for virgin and breeder mice (Tables 3 and 4) indicates that there is a greater degree of variation in the figures for the breeders but the mean, when compared statistically with that of the virgins, is found to be significantly larger for P is less than one percent. Table 5 shows the breeders arranged in two columns. The animals in the right column had had only one litter while those on the left were multiparous. Group comparison between these and the virgins indicates that one pregnancy is sufficient to raise the mammary epithelial iron from virgin to breeder state. Statistical analysis also shows that as far as the results of this small sample are concerned there is no significant difference between the iron content of animals which had born one litter and

those which had born two or more litters.

Tables 6, 7 and 8 show iron analyses of the resting mammary glands of C_{57} Black female mice. The tables are arranged similarly to Tables 3, 4 and 5 and they lead to the same conclusions:

1. C_{57} Black breeders are less consistent in their iron content than the virgins. (Table 6).
2. The mean iron for breeders of this strain is significantly larger than that of the virgins (P is less than one percent). Compare Tables 6 and 7.
3. One pregnancy appears to be successful in raising the stainable intra epithelial iron content from virgin to breeder level. (P is less than one percent - Tables 6 and 8).
4. There is no significant difference in the means between animals which had had one litter and those which were multiparous. (Table 8).

3. Correlation of Histological and Chemical Results:

All histological examinations were done independently without knowledge of the chemical analyses and confirmed by rechecking later at random. The males of the two strains at no time displayed stainable intra epithelial iron.

Table 9 shows the correlation of results between histologically and chemically determined stainable

intra epithelial iron. The small standard errors of the means indicate a remarkable consistency of result for the chemically derived equivalents to the histological iron grades. More-over statistical comparison shows that there is a true difference between the mean iron of any two of the three groups. Reference to Tables 3, 4, 6, and 7, indicate the same result for with few exceptions the histological grades fall in main groups corresponding to the chemically measured iron.

The histological assay is a subjective method, however it is surprising to note the splendid correlation shown in the above experiments, between it and the chemical method. The results are even more remarkable when it is considered that the histological and chemical assays were done on different glands of the small animal.

4. Human Tissue

Table 10 summarizes the results obtained from histological analysis of human tissues which included the male mammary gland. Apocrine sweat glands were observed in all specimens eighteen years of age and over. These glands were recognized as such because of their large lumens, loose coils and because of their position in the subcutaneous tissue. The final criterion by which they were identified was the presence of

stainable intra epithelial iron (Richter 1933). In many instances their ducts were traced into hair follicles, which are believed to be the organs from which apocrine glands develop. There was a conspicuous lack of specimens between the ages of five to eighteen years, so it was impossible to add any information concerning the age of development of the apocrine glands. Specimens younger than five years showed no iron in any of the skin glands in the mammary areas.

The male human mammary gland, which consisted of branching ducts and end bulbs, was found embedded in dense connective tissue. At no time was granular iron observed in any of the ducts or end bulbs of this apparently normal male mammary tissue. However, one specimen (#29) was obtained from a patient who died July 5, 1950, of metastasis from a prostatic carcinoma, and who had been treated for fifteen days during February 1949 with 3 mgms. daily of diethyl stilbesterol. The hypertrophied mammary epithelium was contained within a heavy overgrowth of connective tissue. Numerous end bulbs were present as well as several areas of alveolar development. A fine intra epithelial deposit of iron was observed in the alveolar areas and in many of the end bulbs of this gland.

DISCUSSION

An attempt to use stainable intra epithelial iron as a quantitative measure of the factors responsible for mammary gland development presents peculiar difficulties. Although, as pointed out before, it was by a histological method that Rawlinson ascertained that iron was contained within the cells of the end bulbs and alveoli of mammary epithelium, the histological method as a quantitative measure of this iron and thus of mammary gland development has olvious limitations. Different microscopic fields of the same gland are not necessarily developed to the same degree requiring the observer to average the overall picture.

To meet such criticisms a chemical method of iron micro analysis as outlined above, was used in the hope that it could accurately determine the deposited alveolar iron. This would furnish a useful quantitative tool for the further study of the factors responsible for both normal and abnormal mammary gland development. Many methods of iron micro analysis are available in the literature (Sandell 1944), but the one for total iron determination used in this series of experiments was chosen because it has the advantage of being accurate and is yet simple enough to be practical where only limited chemical equipment is available.

Inbred strains of mice were used in the experiments for the following reasons:

1. It is possible to have very precise data regarding the percentage of animals likely to produce mammary adenocarcinoma, and thus the correlation of cancer incidence to iron content may shed some light on the cancer process.
2. Because the genetic factors being relatively constant, variations due to heredity would be held to a minimum and thus fluctuations in iron content might reflect experimental factors.

To make the results of the iron analysis correspond more closely to the glandular iron alone the two, and sometimes four, lymph nodes associated with the inguinal mammary glands were removed. Separate analysis of the nodes were made and indicated (Table 11) an average iron content of approximately two gamma per animal regardless of sex. The non stainable iron of the mammary pad, or the protein bound iron associated with the fat pad, must be accounted for. The indirect method of making this differentiation by reference to the iron content of the male mammary pad was evolved from the first results obtained from total iron analysis of C_3H mice. These results (Table 12) show that the mammary gland epithelium contains sufficient stainable

intracellular iron to overweigh the protein bound iron because the mean total iron content in the breeders was significantly greater than that of the virgins and the virgins in turn contained a significantly greater quantity of iron than the males. The histological investigations closely paralleled the chemical findings so it was concluded that the total iron was a good approximation of the stainable intracellular epithelial iron.

The total iron was correlated to gland weight in an effort to eliminate the variations due to gland size (Table 12). The inconsistency of results shown in the table was found to be due to a peculiar anatomical relationship whereby the resting mammary gland of the female mouse is contained within a large fat pad. That this large amount of fat could under some circumstances be a significant source of error was shown by the observation that when animals were obviously sick before they were killed, or when post mortem examination showed liver abscesses grossly (presumably from mouse typhoid which was endemic in this colony) the weights of the two inguinal glands were invariably far out of the range of that found in healthy animals. (Table 14). However in these cases as comparison of Tables 12 and 14 shows the total iron found in the sick

animals was well within the normal range for its group. On the contrary, the figures for iron on a gland weight basis were either at the extreme limit of the range for healthy animals of the particular group or very far beyond it. This leads to the conclusion that the weight loss in the glands of a sick animal is largely due to loss of fat substance rather than of gland epithelium. This is born out by the histological findings in that whole mounts of the glands of these animals showed what appeared to be normal amounts of iron for the particular group, but that the gland containing the iron was compressed into a shrunken mass in which the fat was reduced to a minimal amount. It was clear that this last source of error affected the figure for iron per gram of gland much more than the total iron and the possibility was raised that the total iron of the female, considered in conjunction with the male as a base line might be the best simple technique of estimating glandular iron. No claim is made for this as the final method of measuring the iron in question, for it is probable that the development of a chemical method of separation of the protein bound iron plus a correlation to a dry fat free gland weight will produce an accuracy unobtainable with this method. Providing in each experiment, the entire gland epithelium is removed then

the method here outlined eliminates the variation in fat pad weight.

The results of the experiments in two strains of mice indicate a true breeder virgin difference in stainable intra epithelial mammary iron content. There is also a correlation with gland development which provides valuable proof for the original hypothesis that a method of measuring stainable intra epithelial iron would give a quantitative measure of alveolar development. The method should be of use in the investigation of some controversial questions in cancer biology:

1. Whether or not there is a true difference in mammary gland development between high and low tumor strains.
2. Whether or not the mammary tumor agent has an effect on mammary development.
3. Whether or not there is a greater hormonal influence in one strain than another.

Group comparisons (Table 13) indicate that there is a high degree of probability that a true strain difference exists in iron content between C_3H and C_{57} Black virgins and the same degree of probability exists for a true difference between the breeder animals of the same strains. Whether the increased amount of iron found in the C_3H

as compared to the C_{57} Black strain is a reflection of the action of genetic, viral or hormonal influences cannot be determined from the data at hand. C_{57} Black young have been foster nursed on C_3H females, to investigate the action of the milk influence on the architecture of the mammary gland. Investigation of hormonal influences have been commenced by the use of injected estrogens in castrated males, and spayed females. Ovarian grafts have been performed on other males. The results of these experiments will not be ready for some time.

The investigation of iron retention in the human male mammary gland has shown interesting correlation with experimental results obtained by Rawlinson (private communication) in C_{57} Black castrate male mice which had been injected with estrogens. Male controls, both intact and castrated which had been identically treated showed no mammary iron retention, while those treated with estrogens were found to contain stainable intra epithelial iron in the end bulbs. Since the human male mammary gland exhibits very little more than large ducts which do not contain iron, it was considered possible that a gland stimulated by estrogenic treatment to alveolar development might show iron. On this basis a search was made for appropriate human material which

was found in specimen numbered 29. Specimen #29, a human male who had received 3 mgms. of diethyl stilbesterol over a period of fifteen days, sixteen months prior to death showed deposits of blue granular iron in the end bulbs and alveoli when stained by the Gomori technique.

Great controversy exists among anatomists and pathologists over the relationship of the mammary gland to so called apocrine sweat gland epithelium. Numerous hypotheses have been formulated (review of Lee, Pack and Scharnagel 1933) which have been repeated in the literature so often that they now appear to be accepted uncritically even though there is little proof for them. It must be remembered however, that both the mammary gland and so called apocrine sweat glands are epithelial in origin and they have the same apocrine type of secretion. More-over Shultz claims that apocrine sweat glands are only on the primitive milk ridge which extends from the axilla to groin on either side of the trunk. It has long been known that there are areas of pale epithelium (so called because of an apparent inability to take up eosin stain) which are present in the human breast. These cells not only stain similarly to apocrine sweat gland tissue but contain similar lipid fractions

plus the peculiar intra epithelial granular iron deposits common to apocrine glands, Bunting (1948), Richter (1933) has shown that iron is a distinctive mark of apocrine sweat glands. That iron retention occurs in the mammary glands of female mice coupled with the evidence that adenocarcinoma in these glands of the mouse is usually of a cystic variety similar to that found in cysts of human axillary apocrine sweat glands indicate there may be a functional and morphological relationship between these two types. Shultz (1933) refers to Loeschcke as indicating that the apocrine sweat glands are under the influence of sex hormones because these organs develop at puberty. Speert (1942) has produced evidence which strongly suggests that the pale epithelium of the breast in monkeys may appear in response to oestrogenic administration. Whether this is a metaplasia as he claims has not been proven. The finding of iron in the male human mammary gland in response to estrogenic stimutation similar to that produced in the male mouse is strong supporting evidence that there may be a functional and developmental relationship between the mammary gland and apocrine sweat glands.

SUMMARY

1. A chemical technique is described to measure the stainable iron in the epithelial cells of the mammary glands of mice.
2. These results were correlated with microscopic iron estimations and thus with mammary gland development.
3. The technique was used to measure differences in the mammary gland of breeder as compared to virgin mice.
4. The technique was also used to measure differences in mammary gland development between two strains differing in cancer incidence.
5. Iron was found in the mammary gland of the human male after estrogen stimulation.

TABLE 1

Histologically Assayed Stainable Mammary Iron Content Of Breeder As Compared To Virgin Mice 25-50 Wks. Old

Strain	Breeding History	No.	0	1+	2+	3+
C_3H	Breeders	28	0	1	6	21
	Virgin	18	0	11	7	0
C_{57} Black	Breeders	28	0	0	18	10
	Virgins	16	13	3	0	0

0 to 3+ indicates increasing amounts of iron.

TABLE 2

Total Iron Content In Micrograms Of The Two Inguinal Mammary Glands Of Male Mice 25-50 Weeks Old

Data	C_3H	C_{57} Black
Number	29	32
Mean Iron	5.3	3.6
Range Iron	2.3-10	2.7-4.7
Standard Deviation	1.75	0.45
Standard Error of the Mean	0.3	0.1
Mean Gland Weight	0.2107	0.2113
Range Weight	0.0747-0.3557	0.1296-0.3688
Standard Deviation	0.0727	0.0527
Standard Error of the Mean	0.014	0.01

TABLE 3

Iron Content In Micrograms Of The Two Inguinal Mammary Glands Of 25-50 Week Old C_3H Virgin Mice Corrected To Show Intra Epithelial Iron And Arranged In Descending Order

Number	Total Iron	Gland Weight	Tissue Iron	Stainable Intra Epithelial Iron	Histological Grade
472	9.0	0.1250	2.5	6.5	++
474	7.4	0.0806	1.0	6.4	++
557	8.0	0.1123	2.0	6.0	+
463	8.2	0.1272	2.5	5.7	++
482	6.8	0.0909	1.3	5.5	++
505	7.5	0.1107	2.0	5.5	++
475	7.4	0.1170	2.2	5.2	++
480	5.7	0.0746	0.8	4.9	+
461	8.5	0.1619	3.8	4.7	++
558	8.2	0.1636	3.7	4.5	+
473	8.5	0.1723	4.0	4.5	+
484	4.9	0.0749	0.8	4.1	+
498	6.2	0.1140	2.1	4.1	+
556	8.0	0.1746	4.1	3.9	+
481	4.5	0.0754	0.8	3.7	+
516	4.7	0.0987	1.6	3.1	+
504	6.3	0.1541	3.4	2.9	+
515	4.5	0.1580	3.5	1.0	+

Mean Stainable Iron 4.6
Standard Deviation 1.4
Standard Error of the Mean 0.32

TABLE 4

Iron Content In Micrograms Of The Two Inguinal Mammary Glands Of 25-50 Week Old C_3H Breeder Mice Corrected To Show Intra Epithelial Iron And Arranged In Descending Order

Number	Total Iron	Gland Weight	Tissue Iron	Stainable Intra Epithelial Iron	Histological Grade
517	36.7	0.1611	3.7	33.0	+++
518	32.8	0.1307	2.6	30.2	+++
574	32.2	0.2334	6.0	26.2	+++
559	29.2	0.1581	3.6	25.6	+++
551	32.2	0.2681	7.2	25.0	+++
497	24.5	0.1507	3.3	21.2	+++
573	26.3	0.2585	6.9	19.4	+++
585	24.7	0.2660	7.2	17.5	+++
490	19.8	0.1438	3.1	16.7	+++
519	17.7	0.1470	3.2	14.5	++
550	16.0	0.1780	4.2	13.8	+++
487	16.7	0.1391	2.9	13.8	+++
566	18.5	0.2182	5.6	12.9	+++
478	13.9	0.1115	2.0	11.9	+++
619	20.5	0.3242	8.8	11.7	+++
605	18.0	0.2637	7.0	11.0	+++
567	14.5	0.1561	3.5	11.0	+++
476	14.0	0.1606	3.6	10.4	+++
603	16.0	0.2215	5.7	10.3	+++
606	13.3	0.1687	3.9	9.4	+++
572	14.5	0.2180	5.5	9.0	++
555	12.8	0.1747	4.1	8.7	++
569	14.5	0.2750	7.5	7.0	++
620	14.7	0.2821	7.7	7.0	+++
486	8.8	0.1412	3.0	5.8	+++
488	7.1	0.1116	2.0	5.1	++
485	6.9	0.1516	3.3	3.6	++
489	5.1	0.1247	2.4	2.7	+
			Mean Stainable Iron	14.1	
			Standard Deviation	8.0	
			Standard Error of the Mean	1.5	

TABLE 5

Effect Of Multiple Litters On Iron Content In C_3H Mice

Single Litters			Multiple Litters		
Number	Number Of Litters	Iron	Number	Number Of Litters	Iron
476	1	10.4	550	5	13.8
478	1	11.9	551	4	25.0
485	1	3.6	555	2	8.7
486	1	5.8	559	4	25.6
487	1	13.8	574	2	26.2
488	1	5.1	566	2	12.9
489	1	2.7	567	3	11.0
490	1	16.7	569	3	7.0
497	1	21.2	572	3	9.0
517	1	33.0	573	3	19.4
518	1	30.2	585	3	17.5
519	1	14.5	603	4	10.3
			605	3	11.0
			606	3	9.4
			619	5	11.7
			620	2	7.0
		Mean 14.1			Mean 14.1

TABLE 6

Iron Content In Micrograms Of The Two Inguinal Mammary Glands Of 25-50 Week Old C_{57} Black Virgin Mice Arranged In Descending Order Of Intra Epithelial Iron

Number	Total Iron	Gland Weight	Tissue Iron	Stainable Intra Epithelial Iron	Histological Grade
614	7.3	0.2036	3.4	3.9	0
616	6.7	0.1760	2.9	3.8	0
560	7.5	0.2257	3.9	3.6	+
607	5.3	0.1280	1.8	3.5	0
610	4.7	0.1308	1.9	2.8	0
612	5.0	0.1539	2.4	2.6	0
580	5.0	0.1588	2.5	2.5	0
611	4.8	0.1603	2.5	2.3	0
561	5.3	0.1951	3.3	2.0	+
615	4.5	0.1811	3.0	1.5	0
613	6.7	0.2947	5.4	1.3	0
609	5.8	0.2509	4.5	1.3	0
608	5.2	0.2469	4.3	0.9	0
581	4.0	0.2040	3.4	0.6	0
582	3.0	0.1600	2.5	0.5	0
562	5.2	0.2801	5.1	0.1	+
				Mean Stainable Iron 2.1	
				Standard Deviation 1.2	
				Standard Error of the Mean 0.3	

TABLE 7

Iron Content In Micrograms Of The Two Inguinal Mammary Glands Of C_{57} Black Females Corrected To Show, And Arrange In Descending Order Of, Stainable Iron

Number	Total Iron	Gland Weight	Tissue Iron	Stainable Intra Epithelial Iron	Histological Grade
599	14.2	0.1573	2.5	11.7	+++
575	14.3	0.2234	3.9	10.4	+++
594	13.8	0.2125	3.6	10.2	+++
602	12.5	0.1640	2.6	9.9	+++
597	13.8	0.2376	4.2	9.6	++
598	13.0	0.2170	3.8	9.2	++
621	13.7	0.2546	4.5	9.2	++
591	13.7	0.2865	5.2	8.5	++
604	11.8	0.2057	3.5	8.3	+++
625	11.8	0.2143	3.7	8.1	++
595	12.2	0.2418	4.3	7.9	+++
600	11.5	0.2153	3.7	7.8	+++
622	12.3	0.2548	4.5	7.8	+++
588	10.2	0.2156	3.7	6.5	+++
627	11.5	0.2851	5.2	6.3	++
626	8.7	0.1766	2.9	5.8	++
596	9.5	0.2253	3.9	5.6	++
577	8.8	0.1945	3.3	5.5	++
593	8.0	0.1710	2.8	5.2	++
623	7.8	0.1668	2.6	5.2	++
589	8.3	0.1923	3.2	5.1	+ or ++
624	8.7	0.2190	3.8	4.9	++
586	8.2	0.2147	3.7	4.5	++
628	8.8	0.2536	4.5	4.3	++
576	8.7	0.2521	4.5	4.2	++
590	10.5	0.3585	6.7	3.8	++
601	12.0	0.4165	8.8	3.2	+++
587	5.8	0.1826	3.0	2.8	++
			Mean Stainable Iron	6.8	
			Standard Deviation	2.4	
			Standard Error of the Mean	0.5	

TABLE 8

Effect Of Multiple Pregnancies On Iron Content In C_{57} Mice

One Or Two Litters		Three Or More Litters	
Number	Iron	Number	Iron
599	11.7	621	9.2
575	10.4	591	8.5
594	10.2	625	8.1
602	9.9	622	7.8
597	9.6	627	6.3
598	9.2	626	5.8
604	8.3	623	5.2
595	7.9	624	4.9
600	7.8	628	4.3
588	6.5	576	4.2
596	5.6		
577	5.5		
593	5.2		
589	5.1		
586	4.5		
590	3.8		
601	3.2		
587	2.8		
	Mean 7.1		Mean 6.4

Group comparison indicates there is no significance difference in the Means between the two groups.

TABLE 9

Correlation Between Histological Grading Of Iron And Total Iron Found In Both Inguinal Glands

Number of Animals	Histological Grade	Average Iron In Micrograms	Standard Error of Mean
20	3 plus	23.0	2.0
20	2 plus	16.0	1.3
20	1 plus	6.0	0.3

Statistical comparison between any two of the groups gives a probability ratio of less than 1%

TABLE 10

The Presence Of Iron In Apocrine Tissue And Mammary Epithelium Of Human Males Arranged In Descending Order Of Age

Age	Number	A. Glands Present	Iron In A. Glands	Iron In Mammary Epithelium
Premature Babe	14	Nil	Nil	Nil
Few Hours	12	0	0	0
1 Day	20	0	0	0
3 Days	9	0	0	0
4 Days	11	0	0	0
3 Years	21	0	0	0
4 Years	17	?	0	0
18 Years	6	+	+	0
21 Years	19	+	+	0
45 Years	18	+	+	0
48 Years	22	+	+	0
55 Years	4	+	+	0
59 Years	5	+	+	0
63 Years	23	+	+	0
65 Years	7	+	+	0
66 Years	16	+	+	0
68 Years	24	+	+	0
70 Years	13	+	+	0
*77 Years	29	+	+	+
78 Years	10	+	Unstained	0

*Estrogen Treated.

TABLE 11

Iron Content Of Lymph Nodes Associated With Inguinal Mammary Glands Of C_3H Mice

Males		Virgins		Breeders	
No.	Iron in γ	No.	Iron in γ	No.	Iron in γ
479	1.3	464	2.0	476	1.5
491	2.5	472	1.5	478	1.0
492	2.2	473	1.4	485	2.3
493	1.9	474	2.0	486	2.2
500	3.6	475	2.1	487	2.4
509	1.5	480	2.5	488	2.0
510	1.8	481	1.4	489	1.8
512	1.7	482	1.7	490	3.0
513	1.8	484	1.9	497	2.5
514	1.4	498	2.4	518	1.7
		504	2.4	519	1.0
		505	2.2		
10	19.7	12	23.5	11	21.4
Mean	1.97		1.98		1.95

TABLE 12

The Iron Content Of The Two Inguinal Mammary Gland Pads Of C_3H Mice Aged 25-30 Weeks

Group	Number Of Mice	Total Iron γ			γ Iron/gr. Of Gland		
		Range	Mean	S.E.M.*	Range	Mean	S.E.M.*
Males	10	2.3-5.4	4.0	0.3	15.3-72.0	31.3	6.0
Virgin Females	13	4.5-9.0	6.4	0.4	41.0-92.0	60.9	4.6
Breeder Females	12	5.1-36.0	17.0	3.0	41.0-251.0	120.3	19.5

Statistical comparison of total iron content, or of iron per gram of gland, between any two of the three groups of animals gives a probability ratio of less than 1%.

*Standard Error of Mean.

TABLE 13

Comparison Between Iron Content Of Resting Mammary Glands In Two Strains Of Mice

Strain	History	No.	Mean Iron γ	Standard Deviation	Standard Error Of The Mean
C_3H	Virgins	18	4.6	1.4	0.3
	Breeders	28	14.1	8.0	1.5
C_{57} Black	Virgins	16	2.1	1.2	0.3
	Breeders	28	6.8	2.4	0.5

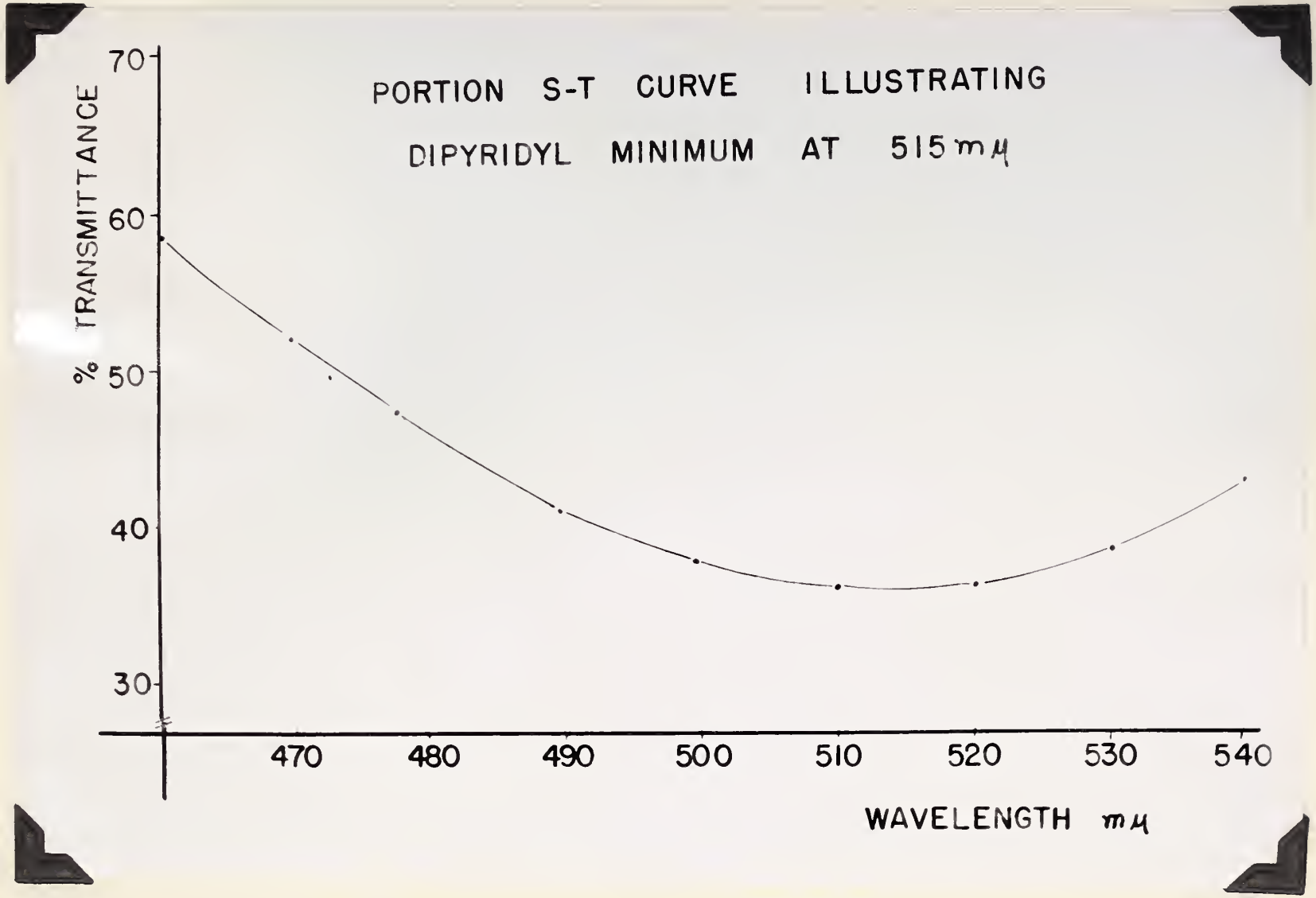
Statistical comparison of mean stainable intra epithelial iron between any two of the four groups of animals gives a probability ratio of less than 1%.

TABLE 14

Effect Of Disease On Gland Weight And Iron Analysis

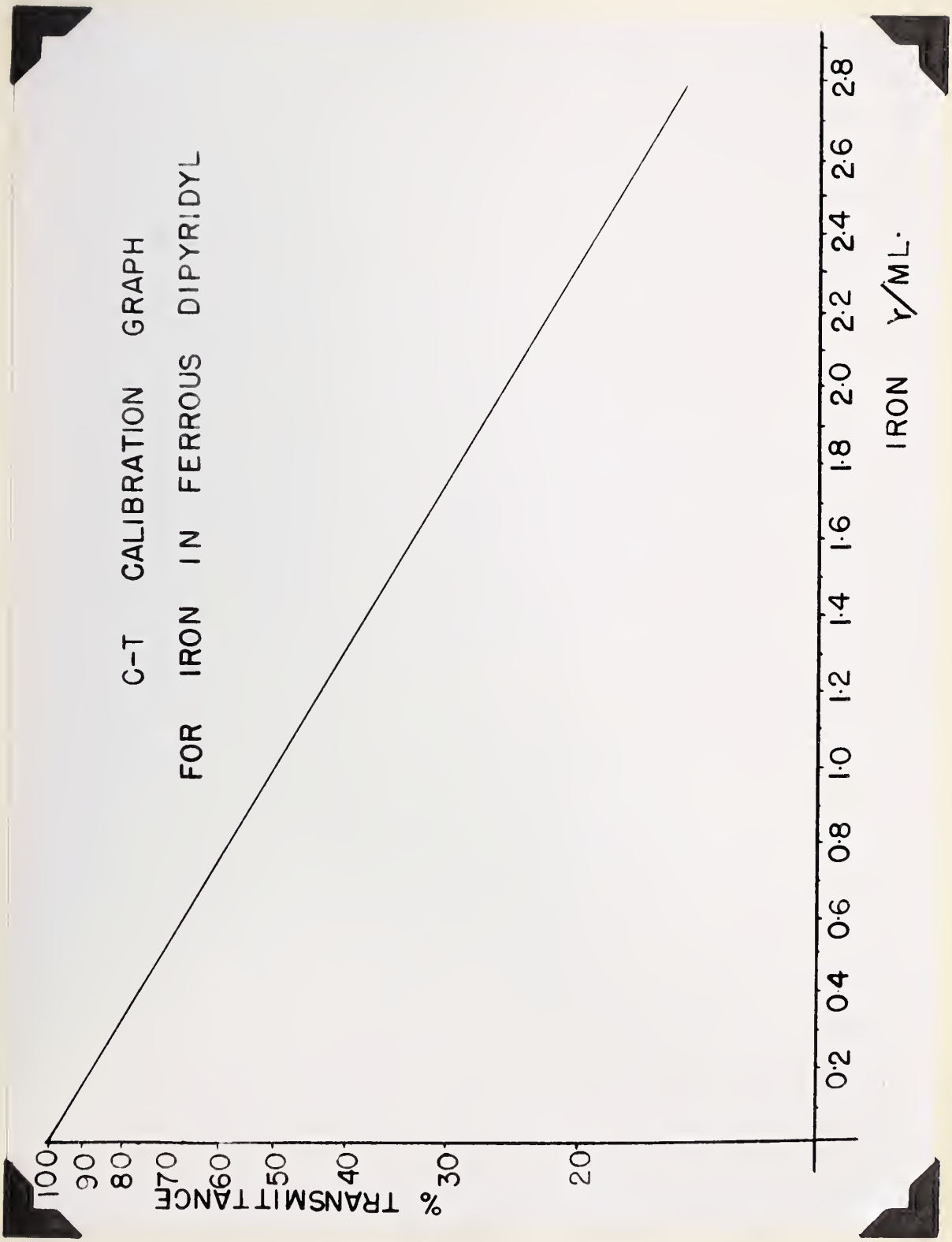
Animal	Gland Weight	Normal Gland Weight		Total Iron	Iron/gm. Gland
		Range	Mean	γ	γ
Male	0.0325	0.0747-0.1794	0.1458	2.5	77.0
Male	0.0180			3.0	167.0
Virgin Female	0.0523	0.0746-0.1723	0.1113	6.5	124.0
Virgin Female	0.0535			5.5	103.0
Breeder Female	0.0402	0.1115-0.1611	0.1395	16.7	415.5
Breeder Female	0.0577			12.7	220.0

Each of the six animals listed in the Tables was excluded from the main experiment because of definite signs of disease: the figures for normal gland weight in grams are included for comparison.

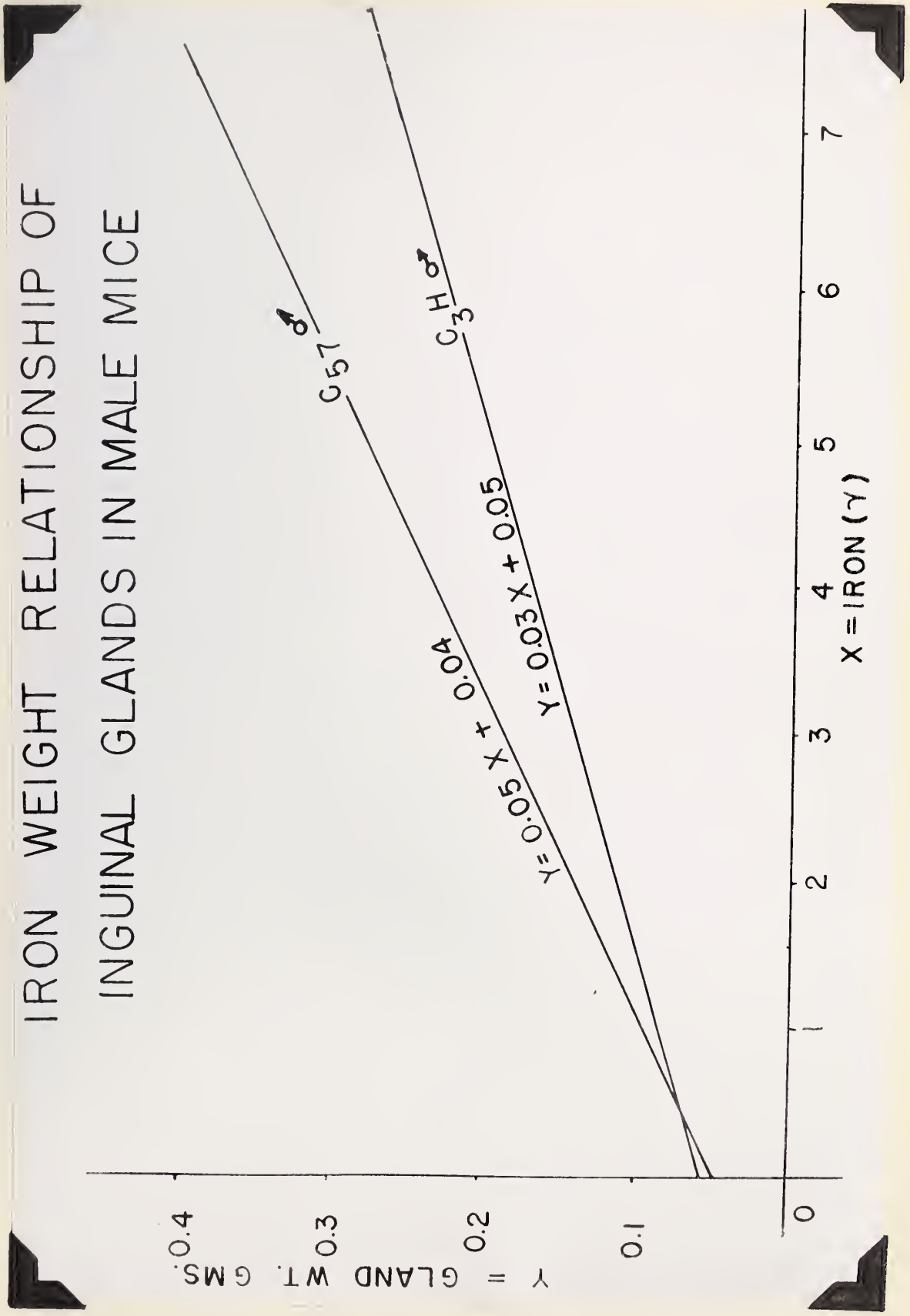

PORTION S-T CURVE ILLUSTRATING
DIPYRIDYL MINIMUM AT 515 mμ
% TRANSMITTANCE
70
60
50
40
30
470
480
490
500
510
520
530
540
WAVELENGTH mμ

Graph 1

Graph 2


C-T CALIBRATION GRAPH
FOR IRON IN FERROUS DIPYRIDYL
% TRANSMITTANCE
100
90
80
70
60
50
40
30
20
0·2 0·4 0·6 0·8 1·0 1·2 1·4 1·6 1·8 2·0 2·2 2·4 2·6 2·8
IRON γ/ML·

Graph 3


IRON WEIGHT RELATIONSHIP OF
INGUINAL GLANDS IN MALE MICE
C57 ♂
C3H ♂
Y = 0.05 X + 0.04
Y = 0.03 X + 0.05
Y = GLAND WT. GMS.
X = IRON (γ)
0.1
0.2
0.3
0.4
0
1
2
3
4
5
6
7

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